Development 136, 3747-3756 (2009) doi:10.1242/dev.038406

Cell competition, growth and size control in the *Drosophila* wing imaginal disc

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We report here experiments aimed at understanding the connections between cell competition and growth in the *Drosophila* wing disc. The principal assay has been to generate discs containing marked cells that proliferate at different rates and to study their interactions and their contribution to the final structure. It is known that single clones of fast-dividing cells within a compartment may occupy the larger part of the compartment without affecting its size. This has suggested the existence of interactions involving cell competition between fast- and slow-dividing cells directed to accommodate the contribution of each cell to the final compartment. Here we show that indeed fast-dividing cells can outcompete slow-dividing ones in their proximity. However, we argue that this elimination is of little consequence because preventing apoptosis, and therefore cell competition, in those compartments does not affect the size of the clones or the size of the compartments. Our experiments indicate that cells within a compartment proliferate autonomously at their own rate. The contribution of each cell to the compartment is exclusively determined by its division rate within the frame of a size control mechanism that stops growth once the compartment has reached the final arresting size. This is supported by a computer simulation of the contribution of individual fast clones growing within a population of slower dividing cells and without interacting with them. The values predicted by the simulation are very close to those obtained experimentally.

KEY WORDS: Wing disc, Cell competition, Growth control, Apoptosis, *Drosophila*

INTRODUCTION

The *Drosophila* imaginal discs have become a very convenient model to study developmental problems. Isolated from most larval tissues and floating in the haemolymph, they are cell populations that grow autonomously and constitute a well-delimited developmental system. Many of their growth parameters, initial and final number of cells, length of the proliferation period, cell division rate, etc. are known after many years of studies (James and Bryant, 1981; Bryant and Simpson, 1984; Madhavan and Schneiderman, 1977). A principal feature of all imaginal discs is the subdivision into anterior (A) and posterior (P) compartments (García-Bellido et al., 1973).

The phenomenon of cell competition was discovered in the wing disc (Morata and Ripoll, 1975). The experiments involved the use of mutations at the *Minute* genes, which encode for ribosomal proteins (Marygold et al., 2007). Heterozygous (*M*/+) flies are viable and fertile and have normal morphology, but suffer a developmental delay in comparison with non-Minute (M^+) flies. The key observation that led to the concept of cell competition was that despite the normal viability of *M*/+ flies, clones of *M*/+ cells were eliminated when generated in M^+ discs. Thus slow-dividing but otherwise viable cells were somehow outcompeted by faster dividing cells. Later work (Moreno et al., 2002) established that slow-dividing cells were eliminated by apoptosis mediated by the Jun N-terminal kinase (JNK) pathway. A key feature of cell competition is that it is a context-dependant phenomenon; it originates from specific interactions between two types of viable cells and leads to apoptosis in one of them.

The phenomenon of cell competition is not limited to the elimination of slow-dividing *M*/+ cells. Viable cells with low activity in the insulin pathway are also eliminated (Bohni et al., 1999). Similarly, cells expressing low levels of *dMyc* (the *Drosophila* homologue of the mammalian proto-oncogene *Myc*) are also outcompeted by cells with higher levels (de la Cova et al., 2004; Moreno and Basler, 2004).

Although cell competition has been studied in detail only in *Drosophila*, it may occur in vertebrates. Mouse cells heterozygous for a riboprotein gene divide slower and are apparently outcompeted by cells with two doses of the gene (Oliver et al., 2004). Furthermore, during rat liver reconstitution hepatic stem cells repopulate the tissue by a process akin to cell competition (Oertel et al., 2006).

The functional significance of cell competition in *Drosophila* is not yet clear. Obviously, the elimination of weaker or slow-dividing cells within an imaginal disc may contribute to the general fitness of the disc. The ability of fast-dividing cells to eliminate slow ones has suggested that cell competition may be a relevant factor in the colonisation of tissues by tumorous cells (Perez-Garijo et al., 2005; Moreno and Basler, 2004; Moreno, 2007).

Another role of cell competition appears to be size regulation; reducing apoptosis in the wing disc results in adult wings of variable size (de la Cova et al., 2004). Moreover, a potential role of cell competition in size control stands from the analysis of M^+ clones generated in *M*/+ discs. These clones do not cross compartment boundaries, but as their cells divide faster than surrounding ones the descendants of a single cell can occupy large portions, often more that 50%, of their compartment, without affecting compartment size. The study of these overgrowing M^+ clones may be useful to analyse the process of size control, because the presence of fast-dividing cells is forcing the control mechanism, which has to cope with cells

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Accepted 14 September 2009

with very different division rates. This is unlike the normal situation, in which all the cells divide roughly at the same rate (González-Gaitán et al., 1994; Milán et al., 1996).

One interpretation of this observation is that the presence of the fast-growing M^+ clones affects $M/+$ cells, which are eliminated in order to build a compartment of normal size: as the M^+ clone grows its cells interact successively with neighbour $M/+$ cells and induce apoptosis in them. The end result of this process would be a compartment of normal size composed of a majority of M^+ cells, just as observed. Recent results have provided some support for this view: overgrowing M^+ clones can induce apoptosis in $M/+$ cells close to their borders (Li and Baker, 2007) (see also Fig. 1).

A related question comes to bear on the mechanism by which M^+ clones overgrow. In the normal situation an M^+ clone induced early in development in an M^+ disc reaches a maximal size of 10^3 cells (García-Bellido and Merriam, 1971), whereas in an $M/+$ disc it often reaches more than 10^4 cells. Li and Baker reported that when cell competition is reduced the degree of overgrowth of the M^+ clones is also reduced: M^+ clones generated in compartments in which cell death is blocked with the caspase inhibitor P35 are smaller than similar clones in control compartments (Li and Baker, 2007). Another way to compromise cell competition is by mutations in the engulfment genes, which are required for the process: when M^+ clones are mutant for some of these genes the degree of their overgrowth is also reduced (Li and Baker, 2007).

In this paper we address the two questions defined above: the role of cell competition in the control of size of the wing disc, and the mechanism of overgrowth of the M^+ clones. Our results show that cell competition has no major role in size control, as compartment size is not altered in the absence of apoptosis, even in the presence of overgrowing M^+ clones. Our findings also show that the overgrowth of M^+ clones does not depend on the killing of neighbouring $M/+$ cells but is the sole consequence of their higher division rate. Computer simulations made on the assumption that $M/+$ and M^+ cells proliferate independently at their own rates make predictions that fit very well with the observed experimental data.

Finally, the results presented in this report provide further evidence for a mechanism of size regulation that functions at the compartment level and that can integrate cells with very different proliferation rates.

MATERIALS AND METHODS

Fly strains and inmunohistochemistry

The fly stocks used were obtained from the Bloomington Stock Center except where indicated: ywhsFlp; RpS17$^-$ ubi-GFP P(w$^+$ FRT2A), RpS17$^-$ ubi-GFP P(neo$^+$ FRT80B), ywhsFlp; ub-GFP P(w$^+$ FRT2A), ywFlp; mwh P(w$^+$ FRT2A), ywhsFlp; mwh P(neo$^+$ FRT80B), ywhsFlp; act>y+>Gal4,UAS-GFP, en-Gal4, UAS-Diap1, UAS-RNAi-Dronc (VDRC), UAS-p35, Histone2-RFF, hh-Gal4 (a gift of T. Tabata, Laboratory of Morphogenesis, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan), UAS-GFP and drpr$^{\Delta 5}$ P(neo$^+$ FRT80B) (gift of N. Baker, Department of Genetics, Albert Einstein College of Medicine, New York, USA).

Fixation and inmunohistochemistry were carried out as described in *Drosophila* protocols. The following antibodies and dilutions were used: phospho-histone 3, 1:400 (Cell Signaling Technology); mouse anti-En 1:50 (Hybridoma Center); rabbit anti-phosphorylated Mad, 1:2000; guinea pig anti-brk 1:1000; rabbit anti-cleaved Caspase 3, 1:50 (Cell Signaling Technology); To-Pro-3, 1:1000 (Invitrogen); rabbit anti-p35, 1:5000 (StrataGene); rabbit anti-dMyc, 1:1000; rabbit anti-Yki 1:1000 (Dong et al., 2007), guinea pig anti-Mer and Ex, 1:2000 (R. Fehon), rabbit anti-dronc1:2000 (Ryoo et al., 2004), rabbit anti-diap1 1:1000 (Wilson et al., 2002). Secondary antibodies were purchased from Invitrogen.

The TdT-mediated dUTP nick-end labelling (TUNEL) assay was performed as described (Udan et al., 2003). Images were taken with Leica TCS SPE and Zeiss LSM510 confocal microscopes.

Induction of M^+ clones

For clones induced 24-40 hours after egg laying (AEL) the heat shock treatment (37°C) was 20 minutes (M^+ clones in M^+ larvae, control clones) and 9 minutes (M^+ clones in M/+ larvae). For clones induced 48 hours before puparium formation (BPF), the heat shock was 13 minutes (control

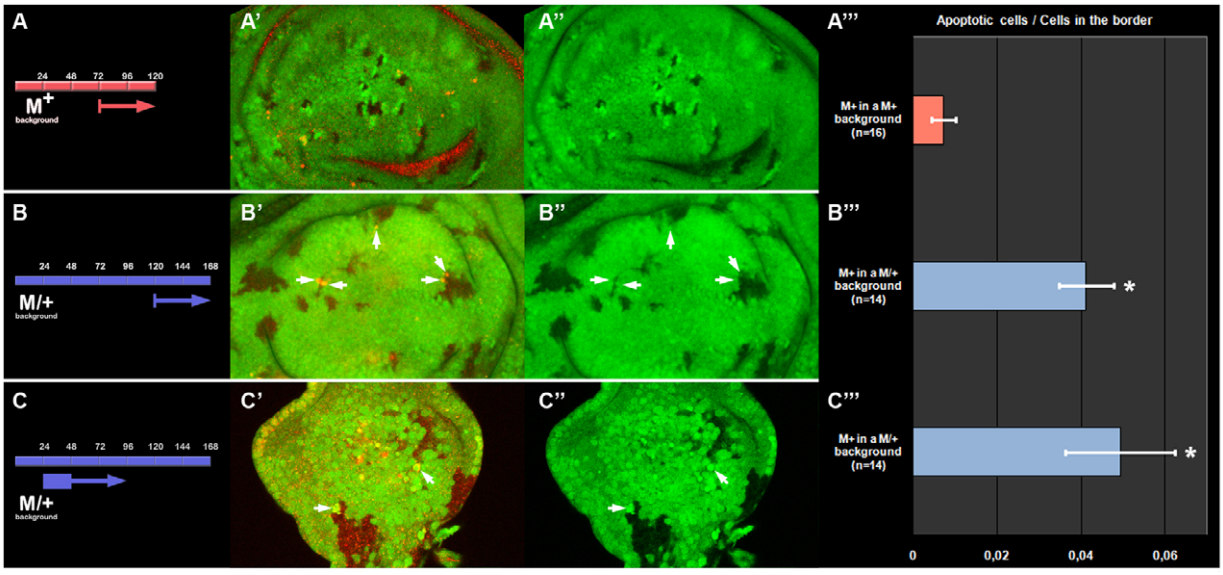

Fig. 1. Cell competition in the border of M^+ clones. (**A-A‴**) M^+ clones growing in an M^+ background 48 hours after clone initiation in third instar discs. (**B-B‴**) M^+ clones growing in $M/+$ third instar discs 48 hours after clone initiation. (**C-C‴**) M^+ clones growing in $M/+$ second instar discs. The left column illustrates, by arrows, the times of clone induction (base of the arrow) and disc fixation (tip of the arrow) with reference to developmental time. The second and third columns show apoptotic cells (red) in discs with clones. The fourth column shows the average frequency of apoptotic cells in the vicinity of M^+ clones. Error bars indicate s.e. *, $P<0.01$.

clones, M^+ in M^+ discs) and 5 minutes (M^+ in $M/+$ discs). For clones induced 72 hours BPF, the heat shocks were 15 and 7 minutes, respectively.

The following genotypes were used. M^+ clones: *ywhsFlp; en-Gal4; RpS17⁻ ubi-GFP P(w^+ FRT2A)/mwh jv P(w^+ FRT2A)*. Control clones: *ywhsFlp; en-Gal4; ubi-GFP P(w^+ FRT2A)/mwh jv P(w^+ FRT2A)*. M^+ clones + diap1: *ywhsFlp; en-gal4/UAS-diap1; RpS17⁻ ubi-GFP P(w^+ FRT2A)/mwh jv P(w^+ FRT2A)*. M^+ clones + p35: *ywhsFlp; en-Gal4/UAS-p35; RpS17⁻ ubi-GFP P(w^+ FRT2A)/mwh jv P(w^+ FRT2A)*. M^+ clones + dronc^{RNAi}: *ywhsFlp; en-gal4/UAS-*dronc^{RNAi}*; RpS17⁻ ubi-GFP P(w^+ FRT2A)/mwh jv P(w^+ FRT2A)*. M^+ drpr⁻ clones: *ywhsFlp; drpr$^{\Delta 5}$ P(neo^+ FRT80B)/RpS17⁻ ubi-GFP P(neo^+ FRT80B)*.

Counting cell number in the wing disc

Larvae from the genotype *UAS-GFP; hh-Gal4/Histone2-RFP* were dissected at prepupal stage, and the discs mounted without coverslip to avoid deformation. Confocal sections per disc were carried out with a Leica TCS SPE microscope separated by 0.6 μm. Images were processed using SVI Huygens Deconvolution and Metamorph software (Molecular Devices) to allow manual counting.

Clone measurements and growth parameters

In clones induced 48 hours BPF, cell number was determined by counting the number of nuclei (positive for To-Pro3) with Photoshop CS3 software (Adobe). In clones induced 24-40 hours AEL, the size (in percentage) is referred to the total compartment size measured in pixels by using WCIF ImageJ software. To measure clone size in adult wings we counted directly the number of *mwh* trichomes of the clones in the wing blade and assumed clone size to be the same in areas in which *mwh* cannot be scored.

Cell doubling times (CDT) in each period of larval development was calculated using the formula CDT=(time after heat shock)/$\log_2$N. For a detailed scheme of clone inductions, see Fig. S3 in the supplementary material. To assess the CDT in wild-type and $M/+$ wing discs, we dissected discs from larva with the following genotypes: *ywhsFlp; act>y⁺>Gal4, UAS-GFP; mwh P(neo^+ FRT80B/mwh P(neo^+ FRT80B)* and *yw hsFlp;act>y⁺>Gal4,UAS-GFP; sc^{s2},RpS17⁻ P(w^+ FRT2A)/mwh P(neo^+ FRT80B)*, respectively.

Computer simulation

The simulation uses a logistic mathematical function based in the CDT of wild-type and *Minute* cells previously obtained. The programming language was BASIC (DarkBasic Software). The growth parameters, final compartment and primordium sizes were implemented into the program. The growth function assigns a different CDT to daughter cells after each division. The length of the CDT depends on the developmental timing, which is a function of the current number of cells and of the remaining cells needed to reach the final size, as imposed by the logistic function. In the wing disc, cell division is asynchronous, and this fact is reflected in the program. For additional information, see the supplementary appendix. The program can be downloaded from the webpage: http://bacterio.cbm.uam.es:8080/scherrera/simulations.zip.

RESULTS

Cell competition, overgrowth of M^+ clones and compartment size control

We investigated the connection between cell competition and the growth of compartment in the wing disc. More specifically, we performed experiments addressed to: (1) measure the ability of M^+ clones to induce apoptosis of $M/+$ cells; (2) compare the growth rate of M^+ clones when they grow in $M/+$ and in M^+ discs, and in conditions in which cell competition is prevented; and (3) test whether the cell competition triggered by the M^+ clones has a significant role in the mechanism of compartment size control.

Induction of apoptosis by M^+ clones in $M/+$ discs

Early third instar $M/+$ and M^+ larvae (see Materials and methods) were heat shocked to generate M^+ clones. We frequently found apoptotic $M/+$ cells near the border of clones (Fig. 1B′,C′), suggesting that there is elimination of $M/+$ cells by cell competition. To ascertain that this apoptosis was actually due to cell competition we compared the number of apoptotic cells at the borders of M^+ clones growing in $M/+$ and in M^+ discs. The latter constitute the control experiment, as in those discs the M^+ clones cannot induce cell competition. We measured the amount of apoptosis in the border of M^+ clones in prepupal discs and also in discs from early third stage larvae. This was because there was the possibility that the amount of cell competition and therefore of apoptosis could be greater in early development, when the cell division rates are higher. As shown in Fig. 1A‴,B‴,C‴ there was a 5- to 6-fold increase in the number of apoptotic cells close to M^+ clones in $M/+$ discs compared with the control. The amount of apoptosis in early discs was not significantly different in discs from different ages. These results provide evidence for cell competition in the borders of the M^+ clones, in support of the previous observation by Li and Baker (Li and Baker, 2007). However, as also indicated in the figure, the number of outcompeted cells appears to be small; only one in 20-24 cells near the clone border showed caspase activity.

We analysed the possible factors behind these events of cell competition by comparing in $M/+$ and M^+ cells the activity levels of pathways involved in disc growth: the Dpp/Brk gradient (Moreno et al., 2002), the Hpo/Warts pathway (Tyler et al., 2007) and the dMyc gene (de la Cova et al., 2004; Moreno and Basler, 2004). Detailed information about these experiments is provided in Fig. S1 in the supplementary material. The result is that we did not detect differences in the levels of these pathways in M^+ and $M/+$ cells.

Growth of M^+ clones in M^+ and $M/+$ discs and in absence of cell competition

Next we measured the growth rate of M^+ clones developing in M^+ and in $M/+$ discs. In these experiments the discs were fixed 48 and 72 hours after clone initiation. The results are illustrated in Fig. 2: the M^+ clones appear to be of the same size whether they grew in an $M/+$ or in an M^+ disc (Fig. 2B,C,H-J). Thus M^+ clones developing in $M/+$ discs grew at the same rate as in an M^+ background. We also measured the sizes of clones in adult wings. For these experiments we heat shocked M^+ and in $M/+$ larvae of various ages. The larvae that reached the prepupal stage (spiracles had been released) after 24±2 and 48±2 hours were separated and allowed to pupate. The wings of the adults that emerged were inspected for *mwh* clones. The data on clone size are included in Fig. S2 in the supplementary material. The size of the 24-hour clones were the same in M^+ and in $M/+$ wings, whereas in the 48-hour series those growing in M/+ wings were slightly larger. Possibly this difference may be due to a longer pupal period in M/+ larvae that allows M^+ clones some extra time. Taken together, these are significant results, for they suggest that M^+ clones grow independently of interactions with neighbouring $M/+$ cells – that is, independent of cell competition.

To explore this idea further, we measured the growth of M^+ clones in compartments in which apoptosis is abolished or greatly reduced. We used the Gal4/UAS method to force expression of several anti-apoptotic factors: the gene *diap1* (*thread* – FlyBase) (Wilson et al., 2002), an RNAi of the apoptotic gene *dronc* (*Nedd2-like caspase* – FlyBase), and the baculovirus p35 gene, which blocks effector caspase activity (Hay et al., 1994). The *en-Gal4* line allows controlled expression of these factors in the posterior compartment, leaving the anterior compartment as control. As shown in Fig. 2, apoptosis, and hence cell competition, was largely abolished in the posterior compartments that contained the *UAS-diap1*, the *UAS-dronc^{RNAi}* or the *UAS-p35* construct.

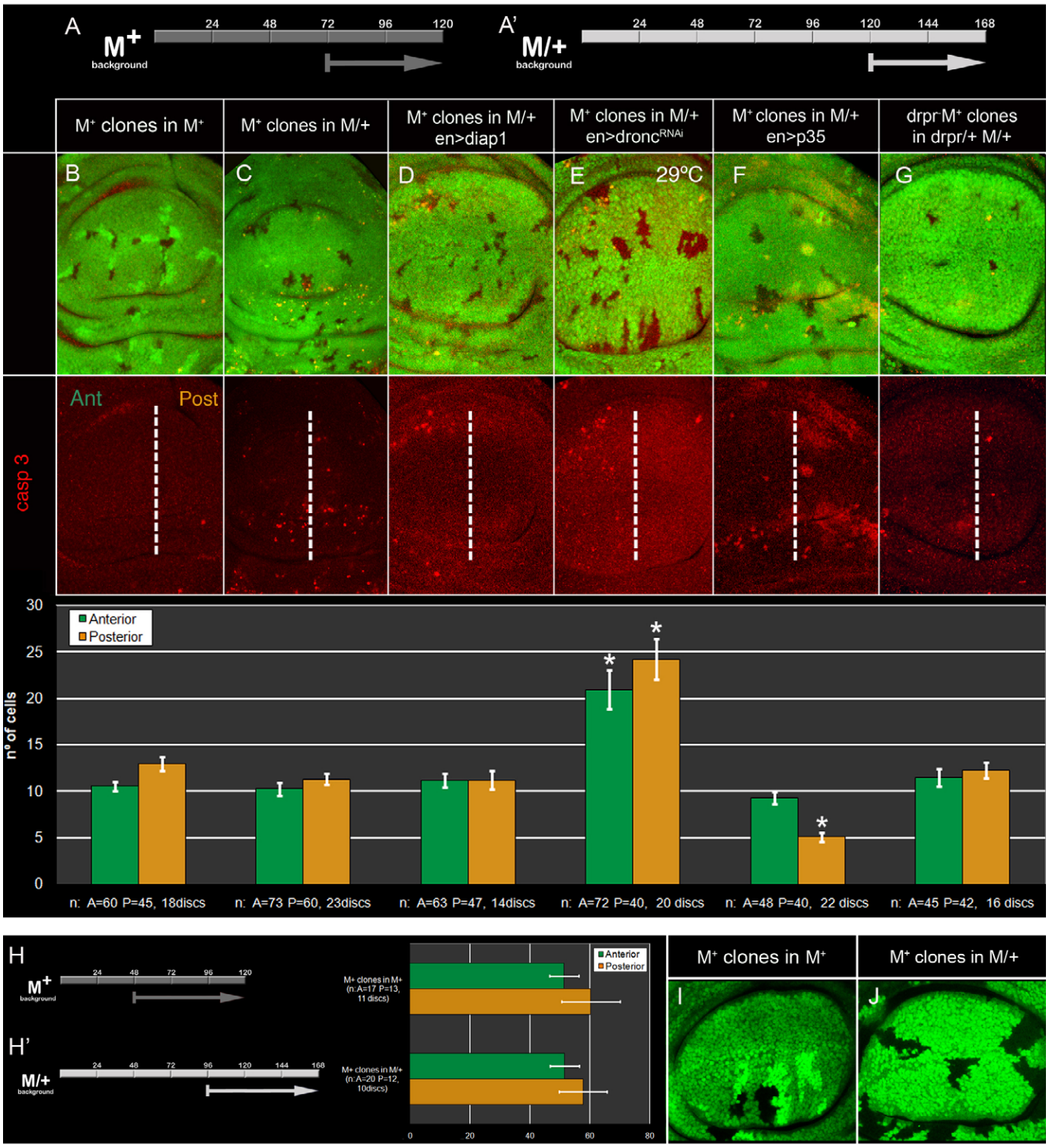

Fig. 2. Size of *M*$^+$ clones 48 and 72 hours after clone initiation in different backgrounds. (**A**,**A'**) The time of clone initiation and disc fixation in *M*$^+$ and *M*/+ discs, respectively, in the 48 BPF clones. (**B-G**) The different genotypes: (B) *M*$^+$ clones growing in *M*$^+$ discs; (C) *M*$^+$ clones growing in *M*/+ discs; (D) *M*$^+$ clones growing in *M*/+ *en>diap1* discs; (E) *M*$^+$ clones growing in *M*/+ *en>dronc-RNAi* discs; (F) *M*$^+$ clones growing in *M*/+ *en>p35* discs; (G) *drpr*$^-$ *M*$^+$ clones growing in *drpr*$^+$ *M*/+ discs. The top row shows discs with clones. The middle row shows caspase activity in the same discs as in the top row. Note that there is very little apoptosis in B and that in the C-E columns the apoptotic cells are frequently associated with the clone borders. In the posterior compartments of the discs in D and E columns, there is very little apoptosis associated with the *M*$^+$ clones. In column G, apoptosis is much reduced in both the anterior and the posterior compartments. The bottom row shows the average size of clones in the A compartment (green) and in the P compartment (orange). The number of clones in the A and P compartments and the number of discs examined are also shown. (**H**,**H'**) Induction of 72 hours BFP clones in *M*$^+$ and *M*/+ discs. The average sizes of the clones are shown to the right of the panel. (**I**,**J**) Examples of *M*$^+$ clones in *M*$^+$ and *M*/+ discs. Error bars represent s.e. *, *P*<0.01.

We also interfered with cell competition by examining M^+ clones that are defective in engulfment gene activity. It has been shown that the interactions between 'winner' and 'loser' cells leading to cell competition require activation of engulfment genes in the winner cells (Li and Baker, 2007). Mutations at *draper* (*drpr*) appear to be effective in reducing cell competition: M^+ $drpr^-$ clones are much less able to outcompete *M/+* cells than are M^+ $drpr^+$ clones. In our experiments we also found a reduction in the amount of competition in the borders of M^+ $drpr^-$ clones: whereas in M^+ $drpr^+$ clones there was an average of one apoptotic cell in 24 in the clone borders, in M^+ $drpr^-$ clones there was one in 55. In the control (M^+ clones growing in M^+ discs) the number of apoptotic cells in the border was one in 143.

The results concerning the effect of apoptosis inhibition on the growth of M^+ clones are illustrated in Fig. 2D-G: the size of M^+ clones in posterior compartments of genotype *en>diap1* (Fig. 2D) or *en>dronc*^{RNAi} (Fig. 2E) was the same as in anterior compartments. The overall clone size (in anterior and posterior compartments) in the *en>dronc*^{RNAi} experiment was higher because the larvae were kept at 29°C to obtain a more effective reduction of *dronc* activity. Because the discs were fixed 48 hours after clone initiation, as in the other experiments, this resulted in bigger clone size. In the case of the $drpr^-$ experiment (Fig. 2G) the M^+ clones were of the same average size as M^+ $drpr^+$ clones (Fig. 2C). We found a significant size difference only in *en>p35* discs, in which M^+ clone size was smaller in the posterior than in the anterior compartments (Fig. 2F). This result is in agreement with the previous observation by Li and Baker (Li and Baker, 2007) and is discussed below.

The preceding results (except those from of the *en>p35* experiment) suggest that the growth of M^+ clones is not significantly affected by their ability to outcompete neighbouring *M/+* cells. However, the clones generated in the previous experiment were relatively small and grew only during a limited period of time. We then checked whether early M^+ clones, which occupy very large portions of the compartment, make use of this ability for their overgrowth.

A precise estimate of the average contribution of single early M^+ clones was lacking. To address this question we generated early M^+ clones (24-40 hours AEL). As the first divisions start at about 40 hours (Madhavan and Schneiderman, 1977), these clones should appear after the first division. The larvae were given a short heat shock (9 minutes) in order to have a low frequency of clones and thus to avoid having more than one clone per compartment. Under these conditions there is an average of 0.5 clones per disc. The results are shown in Fig. 3. For M^+ clones growing in M^+ compartments the average size was around 3-4% of the respective compartment (Fig. 3B,C), whereas M^+ clones in *M/+* compartments occupied an average of 48% in the anterior and 53% in the posterior compartment (Fig. 3B,C). In these experiments the M^+ clones growing in *M/+* discs were much bigger than those growing in M^+ discs, in spite of having a similar proliferation rate, because the length of the proliferation period is longer in *M/+* larvae.

If the overgrowth of the M^+ clones occurs at the expense of the elimination of *M/+* cells it would be expected that the absence of apoptosis should make these clones smaller. As indicated in Fig. 3, the mean size of M^+ clones in the anterior compartments – where apoptosis is not compromised – is the same as in the posterior compartments. This result does not support the argument that M^+ clones overgrow due to elimination of *M/+* cells. We found a difference in clone size between the anterior and posterior compartment only when cell death was blocked with P35 in which posterior M^+ clones were smaller than those in the anterior compartment. Although we do not have a clear explanation for this difference, in *en>p35* experiments the last step of apoptosis, that is, the function of the effector caspase Drice (Ice – FlyBase), was blocked (Hay et al., 1994). Under these conditions the *M/+*; p35 cells that initiate apoptosis become 'undead' cells, which emit mitogenic signals such as Dpp and Wg (Huh et al., 2004; Pérez-Garijo et al., 2004; Ryoo et al., 2004) (Fig. 4D-D″). In the experiments interfering with apoptosis by overexpressing *diap1* or inhibiting *dronc*, undead cells were not produced because they require normal activity of *dronc* and subsequent inhibition of *drice* function (Kondo et al., 2006; Martín et al., 2008). This ectopic signalling may promote proliferation of nearby *M/+* cells, which may provide a larger contribution to the final compartment. Interestingly, the posterior compartments containing p35 were bigger than those without it (Fig. 4B), probably the result of the ectopic signalling generated by the undead cells.

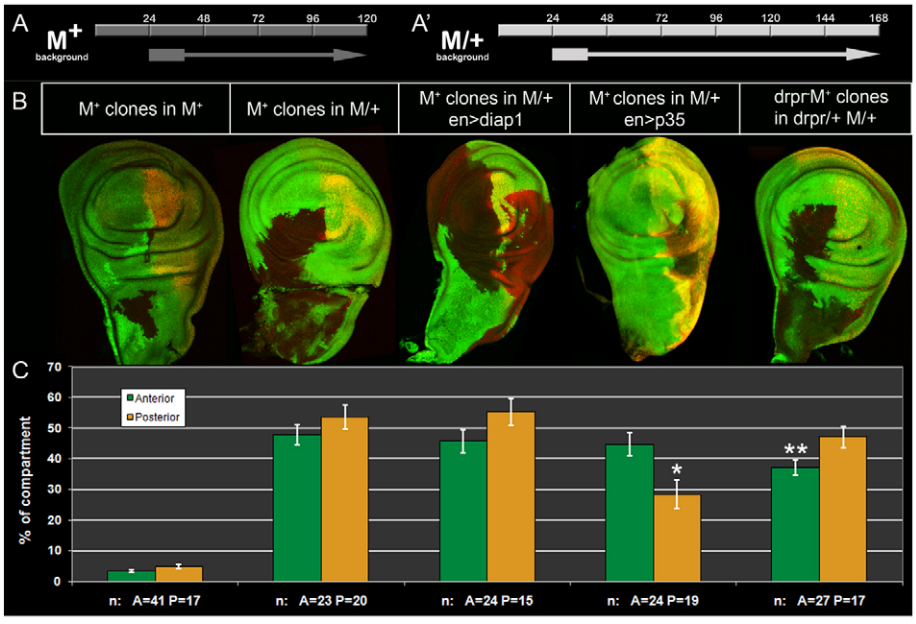

Fig. 3. Average size of individual early M^+ clones in different genotypes. (**A**,**A′**) The time of clone initiation and disc fixation in M^+ (A) and *M/+* (A′) discs. (**B**) Discs with unique clones labelled by the loss of GFP. The genotype of the clones is indicated above the discs. Posterior compartments are labelled in red with anti-Engrailed, except the *M/+ en>diap1* disc, which is stained with anti-Diap1, and *M/+ en>p35*, which is stained with anti-P35. (**C**) The average clone size. The number of clones studied for each genotype in the A and P compartment is indicated. Error bars indicate s.e. *, $P<0.01$; **, $P<0.05$.

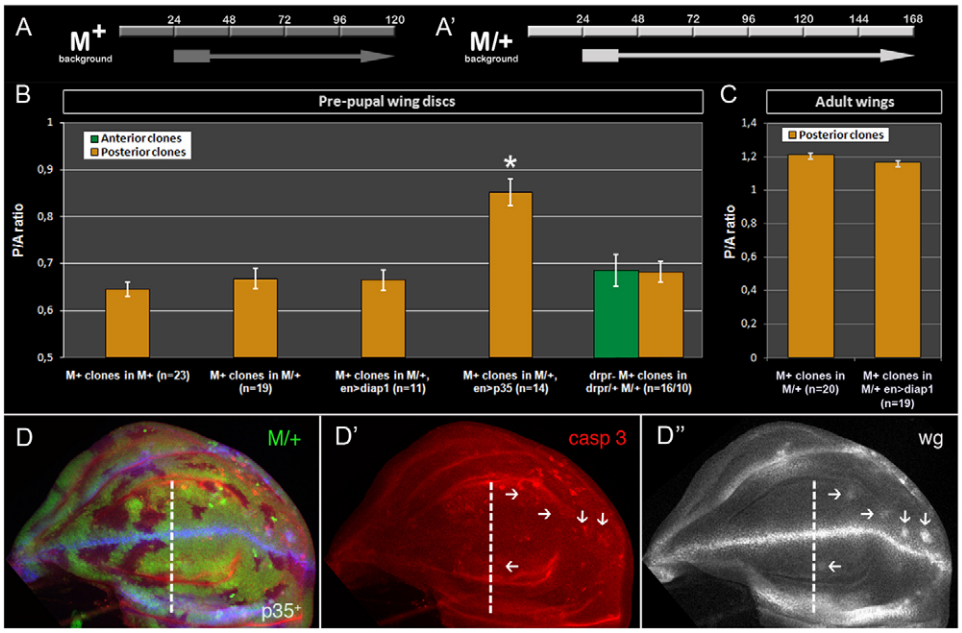

Fig. 4. Relative size of posterior compartments containing M^+ clones. (**A**,**A′**) The time of clone initiation and disc fixation in M^+ (A) and $M/+$ (A′) discs. (**B**) P/A ratio of wing discs of various genotypes (indicated below the bars) containing M^+ clones in the posterior compartment. There is no significant difference in the ratio except in the case of M^+ clones in posterior compartments containing the *p35* gene. (**C**) P/A ratio measured in control and *en>diap1* adult wings. (**D-D″**) The wing pouch region of an *en>p35* disc containing M^+ clones. Note the presence of cells ectopically expressing the *wg* gene, which probably causes additional growth in the compartment. Error bars indicate s.e. *, $P<0.01$.

Role of cell competition in the control of compartment size

The observation that M^+ clones often induce apoptosis in neighbouring $M/+$ cells may suggest that cell competition could act to eliminate the excess of cells that would be produced in compartments containing overgrowing M^+ clones. It follows from this argument that the abolition of apoptosis, and hence of cell competition, in $M/+$ compartments harbouring early M^+ clones should produce an excess of growth due to the contribution of the rescued $M/+$ cells.

We checked this possibility by measuring the P/A size ratio of discs and also adult wings in which the apoptosis-inhibited posterior compartments contain early M^+ clones. We also measured the size of *drpr*$^-$ mutant discs in which cell competition is compromised. In discs of genotype *en>diap1* the P/A size ratio was normal, just like in the wild type; in mature discs it was 0.66, whereas in adult wings it was 1.16 (Fig. 4B,C). The different values of P/A in discs and adult wings derive from the method used to estimate them. For the discs we considered the whole structure, whereas for the adult wings we measured only the wing blade, in which the anterior and posterior compartments are of roughly the same size.

Similarly, the P/A size ratio of *drpr*$^-$ discs containing M^+ clones only in the anterior or only in the posterior compartment (Fig. 4B) was also like that in wild-type discs. The only departure from the normal situation is the genotype *en>p35*, in which the P/A ratio was significantly increased. We believe this is because of the presence of ectopic signalling by undead cells (Fig. 4D-D″).

The clear conclusion from these experiments is that under these conditions cell competition does not play a significant role in size control. These results also illustrate that fact that the size control mechanism can cope with very different cell proliferation rates within a compartment: it functions equally well in compartments in which all the cells divide at the same rate, the normal situation, or in compartments in which M^+ clones occupy most of it.

A computer simulation of the growth of M^+ clones

All the preceding results suggest that in the wing disc clones with different division rates grow autonomously; their contribution to the final structure is exclusively determined by their growth rate. The overgrowth of M^+ clones would simply be due to their higher growth rate, without having to invoke interactions between M^+ and $M/+$ cells. The only limitation would be that the final size of the compartment is fixed, as imposed by the control mechanism. If the contribution of the M^+ and $M/+$ cells in a mosaic disc were the automatic result of their respective growth rates, it should be possible to build a computer simulation that mimics the experimental results.

Basic growth parameters of M^+ and $M/+$ discs

For the simulation it was necessary to ascertain the basic growth parameters of M^+ and $M/+$ wing discs. In particular, the cell proliferation rate (as indicated by the CDT) at different developmental periods, and the initial and final number of cells.

We addressed the first question by inducing in M^+ and $M/+$ larvae Flp-out clones marked with GFP (see Materials and methods) at defined developmental stages and fixing the discs at precise times after clone initiation. For M^+ larvae we considered the 40-72, 72-96 and 96-120 periods and for $M/+$ the 40-72, 72-96, 96–120, 120-144 and 144-168 periods (Fig. 5A and see Fig. S3A,B in the supplementary material). To have precise timing of the clones, the egg-laying periods were very short, 2-4 hours, and the heat shock was 7 minutes (to avoid having too many clones per compartment that may cause merging). The heat shocks were administered to larvae of 40, 72 and 96 hours of age for M^+, and 40, 72, 96, 120 and 144 hours for the $M/+$. The discs were fixed at various times after the heat shock (see Fig. S3A in the supplementary material).

We found that in early M^+ discs (40-72 hours AEL) the initial CDT was about 5.5–5.7 hours. This value increased as larval development proceeded up to a value of about 30 hours in the last division, just before pupariation (Fig. 5A and see Fig. S3A,B in the supplementary material). The total number of cell divisions was about 9.1, which is in agreement with previous data (García-Bellido and Merriam, 1971). In early $M/+$ discs (40-72 hours AEL) the initial parameters were quite different, with a CDT of 11 hours, whereas the last division took about 35 hours. The greater difference in cell division rate between M^+ and $M/+$ discs appears to occur during the early proliferation phase.

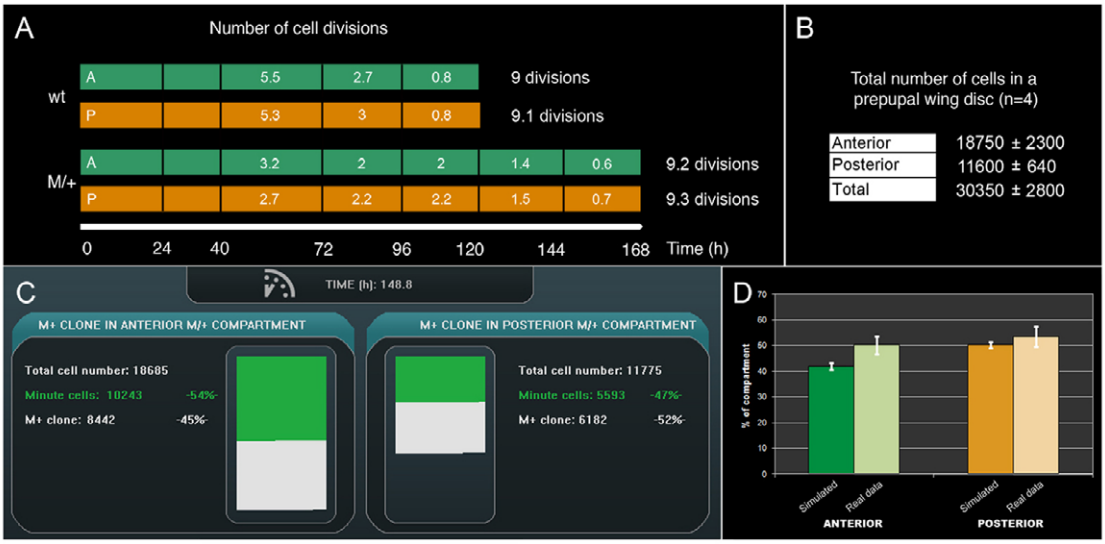

Fig. 5. Proliferation rate during development, final number of cells and results of the computer simulation. (**A**) The number of divisions performed in 24-hour periods during the proliferation phase of disc development in wild-type and *M/+* larvae in anterior (green) and posterior (orange) compartments. Note that the majority of divisions take place during early disc development. (**B**) Estimates of the final cell population in the anterior and the posterior compartment. For details of the calculation, see Fig. S3 in the supplementary material. (**C**) Illustration of single simulation events in the anterior and posterior compartments. (**D**) Comparison of the average sizes of clones in the anterior and posterior compartment as predicted by the computer simulation and as obtained in real experiments. Error bars indicate s.e. In all cases, $P>0.05$.

The initial number of cells in the wing disc was measured by Madhavan and Schneiderman by direct counting in first instar larvae, and they found 37-38 cells (Madhavan and Schneiderman, 1977). As in the embryonic primordia, the P/A compartment ratio was 0.5, which would indicate about 25 cells for the A and 12–13 for the P compartment. Their estimate of the CDT of the first two divisions was 6-7 hours, similar to our measurement. We calculated the initial cell number by an indirect measurement based on the final number of cells in the prepupal disc (see below).

Previous measurements of the final number of cells suggest a value around 50,000 cells (García-Bellido and Merriam, 1971; Graves and Schubiger, 1982). We counted directly the number of cells by using a nuclear-RFP under a ubiquitous promoter. We mounted four prepupal wing discs and made a careful 3D reconstruction using a deconvolution program (for details see Materials and methods). We were able to discriminate each nuclei of the wing imaginal disc with high accuracy (see Fig. S3C-G in the supplementary material). Then, we divided the epithelia in squares and made an estimate of cell number. The four discs yielded similar values and we obtained an average number of 30,350±1400 cells (Fig. 5B) of which 18,750±1160 belonged to the A compartment and 11,600±320 to the P compartment. Considering that the total number of cell divisions was 9.1, it suggests that the initial anterior polyclone would have 34 cells and the posterior 21.

Predictions of the computer simulation and comparison with experimental results

The simulation program was built with the following assumptions: (1) M^+ and *M/+* cells divide at their own rate; (2) each compartment will stop growing once it has reached the stereotyped final number of cells; (3) cell divisions are asynchronous; (4) during development, cells adjust their CDT in accordance with the size of the compartment at each moment and the stipulated final size, as imposed by the logistic function that governs growth of animal tissues (Bryant and Simpson, 1984).

A key simulation would be that of a compartment containing one M^+ cell and the remainder *M/+*. It would represent the expected contribution of a single M^+ clone growing together with, but independent of, the surrounding *M/+* cells. Given the stochastic conditions of the cell cycle used in the simulation, every event renders slightly different results. Examples for a single M^+ clone growing in an anterior and in a posterior compartment are shown in Fig. 5C. The computer simulation consistently gave a figure of about 45-50% as the average contribution of a single M^+ clone in both cases (Fig. 5D), which fits well with the experimental results. This finding strongly supports the conclusion that *M/+* and M^+ cells proliferate independently. The computer simulations predict that very few M^+ clones would cover a large portion of the disc (see Fig. S4A in the supplementary material). This probably accounts for the cases reported in the literature of M^+ clones covering the majority of the compartment. For clones initiated later in development (72 hours BPF), the computer simulations also predict values that turned out to be very similar to those obtained experimentally (see Fig. S4B in the supplementary material).

We also performed computer simulations considering different parameters of cell death during development: the number of dying cells and the time needed for apoptotic cells to disappear after they show caspase activity. The results are shown in Fig. S4C in the supplementary material. They can be summarised by saying that only very high levels of apoptosis would have a significant effect on M^+ clone size. For example, assuming that apoptosis affects one out of every three M/+ cells surrounding M^+ clones and that it takes the cells 8 hours to disappear, the average M^+ clone size would be 70.5% of the compartment. By contrast, the measurements of apoptosis associated with M^+ clones oscillated between 1/20 and 1/24 (Fig. 1). We also modified other parameters, such as the time needed for the apoptotic cells to disappear, also shown in Fig. S4 in the supplementary material.

Compartments containing M^+ clones are ahead in development

The computer simulation also predicts that *M/+* compartments containing an M^+ clone should reach the final arresting size earlier than compartments constituted only by *M/+* cells. If an *M/+* compartment is expected to finish development by 168 hours, the average of several events of compartments with individual M^+ clones is about 148 hours, a difference of 20 hours. As a consequence of the faster growth rate of the M^+ clones, those compartments should be ahead in their developmental progression and hence would arrest growth earlier.

One way to test this prediction is to compare the developmental progression of compartments of the same disc in which one compartment contains an M^+ clone and the other does not. To monitor the progression we used the activity levels of the genes *vestigial* (*vg*) and *wingless* (*wg*), the expression patterns of which evolve during development so they can be used to compare relative developmental stages (see Martin and Morata, 2006). We dissected discs from early or mid-third instar *M/+* larvae and considered only those cases in which one disc contained an M^+ clone and the other wing disc of the same larva did not. The latter was used as control. As can be seen in Fig. 6A,A′, the expression of *vestigial* in the anterior compartment of a wing disc containing an M^+ clone was at that stage more advanced than in the posterior compartment of the control disc. Similarly, *wg* expression was more advanced in the compartment containing an M^+ clone (Fig. 6B,B′). These results indicate that the presence of an M^+ clone accelerates the development of the compartment. This effect should be reflected in the time needed to reach the final arresting size.

Moreover, these compartments containing large M^+ clones can be used to study the cell autonomy of the compartmental expression of developmental genes such as *vg* or *wingless* (Kim et al., 1996; Ng et al., 1996; Whitworth and Russell, 2003; Williams, 1994). As illustrated in Fig. 6D, the border of some M^+ clones intersect the vg domain, splitting it into M^+ and *M/+* territories. It can be observed that the developmental stage of *vg* expression was the same in the M^+ and *M/+* territories. This is an indication that, like the mechanism governing compartment size, the process controlling developmental progression does not operate cell-autonomously, but at the whole compartment level. It may well be that it is the same process controlling the developmental progression of the disc and that that arrests growth once the final compartment dimension has been reached.

DISCUSSION

The main objective of this work was to study the role of cell competition in regulating growth and size in the wing disc of *Drosophila*. As the disc is composed of two (A and P) compartments, which behave as independent units of size control (Martin and Morata, 2006), our experiments refer to mechanisms operating within compartments.

To make some precise statements about the growth dynamics of the disc we have calculated several developmental parameters, some of which had not been described in detail in previous publications. According to our data, the wing disc starts growth with about 55 cells, of which 34 would belong to the A and 21 to the P compartment. The final cell number is around 30,000 (about 19,000 A and 11,000 P). The total number of cell divisions is about 9.1. These estimates coincide well with previous ones (García-Bellido and Merriam, 1971; Graves and Schubiger, 1982), although the final number of 30,000 cells is somewhat lower than previous measurements.

We show here, in agreement with previous results (James and Bryant, 1981; Neufeld et al., 1998), that the growth rate of the wild-type wing disc changes markedly through development: during the second larval period M^+ cells divide at about 5.5-5.7 hours per cycle, then the length of the cell division cycle increases as development progresses, and in the second half of the third larval period it is as high as 30 hours (Fig. 5). Thus most of the growth occurs during the second and early third larval period. We find a similar growth pattern in *M/+* discs, the CDT of which increases from 10-12 hours in the second larval period to 34-40 at the prepupal stage. It is not surprising that the major difference between wild-type and *M/+* discs occurs during the early periods. Possibly the metabolic demand is greater in fast-proliferating cells and therefore the limitation in protein synthesis of *M/+* cells is more noticeable.

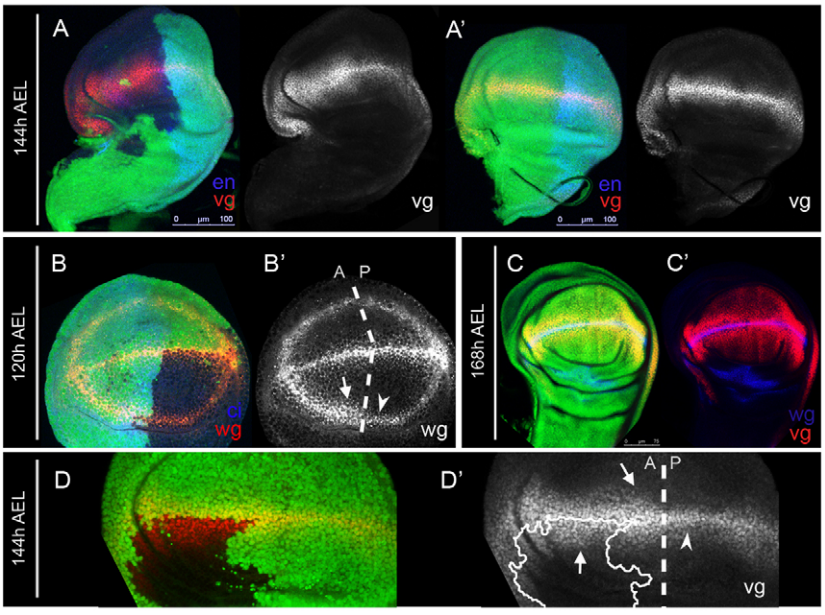

Fig. 6. Effect of the presence of an M^+ clone on the developmental progression of a compartment. (**A**,**A′**) Two wing discs from the same mid-third instar larva, fixed 144 hours after egg laying. They are stained for *engrailed* (blue) and *vestigial* (red). The A disc contains an M^+ clone (marked by the loss of GFP) in the anterior compartment, whereas the A′ disc does not. Note that *vg* expression is more advanced in the anterior compartment. The expression of *vg* in the posterior compartment of the A disc is like that in the A′ disc. (**B**,**B′**) Early third instar wing disc, stained for *wg* activity, containing an early M^+ clone in the posterior compartment. The development of the posterior compartment (arrowhead) is more advanced than in the anterior compartment (arrow), as indicated by the more sharply defined *wg* expression. (**C**,**C′**) An *M/+* prepupal disc showing mature expression of *wg* (blue) and *vg* (red). (**D**,**D′**) Wing disc harbouring an M^+ clone in the anterior compartment that splits it into M^+ and *M/+* territories (arrows). Note that *vg* expression is more advanced in the anterior compartment, but within the compartment it is equally advanced in the M^+ and *M/+* territories.

Cell competition and growth control in the wing disc

Normally there is little apoptosis in the wing disc (Milan et al., 1997); therefore, cell competition cannot have a major role in normal circumstances. Nevertheless, it may be a safeguard mechanism to eliminate abnormal cells or to deal with unusual situations such as cells with different division rates, which may interfere with the growth of the disc. We have examined the significance of these events of cell competition in the overgrowth of M^+ clones and in the control of compartment size.

The fact that M^+ clones growing in *M/+* discs can reach an average size more than ten times their normal size (when they grow in a M^+ disc) while not altering the final size of the compartment, suggested that both clone overgrowth and size control may depend on cell competition. In this view the M^+ clones would overgrow at the expense of the elimination of neighbouring *M/+* cells. Moreover, the removal of the latter would ensure that they do not produce progeny that would give rise to an excess of cells in the compartment.

However, our experiments indicate that cell competition does not play a significant role in these processes; in the absence of cell competition, the M^+ clones are able to overgrow as much as in the normal situation (Figs 2 and 3). Besides, the size of compartments is not affected by the presence of these clones, even though they can occupy the larger part of the compartment.

In our view, the key element to explain these results is the mechanism that controls the overall size of the compartment and that arrests growth once it has reached the final dimension. This mechanism would function without regard to the lineage of the cells or of their relative contribution to the final structure. It would also function autonomously in each compartment (Martin and Morata, 2006).

Let's consider a compartment containing M^+ and *M/+* cells from early in development. The cells proliferate at the rate dictated by their metabolic activity, according to their *Minute* genotype, and their division rate is not affected by interactions with neighbour cells. Because of their proliferation advantage the M^+ cells occupy a large part of the compartment. In principle, if the *M/+* cells were to proliferate at their normal rhythm for the whole duration of the *M/+* larval period, the sum of the contribution of the M^+ and *M/+* cells would produce an excess of tissue. The reason why this is not the case is that the size control mechanism arrests growth as soon as the final size has been reached. In the presence of a large M^+ clone the final arresting size of the compartment is reached *earlier* than in a one entirely made of *M/+* cells. For this reason the *M/+* cells contribute less than they would have in the absence of a M^+ clone. That is, the developmental delay associated with the *M/+* condition is partially abolished by the presence of the M^+ clone. This is predicted by the computer simulation and is supported by our results. Using the expression of the *vg* and *wg* genes to monitor the developmental stage of the compartment, we find that compartments with M^+ clones are ahead and are therefore expected to reach the final arresting size earlier than those that are entirely *M/+* (Fig. 6).

The existence of a non-cell-autonomous mechanism governing the growth of the compartment is also suggested by our observations about *vg* expression in compartments containing M^+ clones. As shown in Fig. 6D,D′, M^+ clones can sometimes split the vg domain into M^+ and *M/+* territories. The significant finding is that both territories show the same pattern of expression. This very strongly suggests that the control of *vg* expression is determined by an overall mechanism probably measuring the size of the compartment in each moment and regardless of the individual lineages.

The physiological role of cell competition

What, then, is the role of cell competition? Cell competition results from interactions between two types of viable cells and causes the elimination of one of them. That is, it is a mechanism to remove viable cells that are not developmentally adapted to the growing tissue. Unlike other situations that cause apoptosis, the 'loser' cells in the competition process are not necessarily damaged; they are poor competitors. In the cases reported here, it is the relatively slow proliferating cells that are eliminated, which may have a beneficial effect on the general fitness of the disc (Moreno et al., 2002). Nevertheless, there may be other safeguard functions of greater biological significance. Cell competition may be instrumental in removing viable but developmentally abnormal cells, which, for instance, do not interpret developmental cues correctly (Milán et al., 2002). This would include tumour or transformed cells that may arise in development. The process would ensure that tumour cells would normally be outcompeted by non-tumour cells. In certain circumstances, however, the acquisition by the latter of some additional property may turn tumour cells into super-competitors, thus reversing the situation. It has been argued that cell competition may be a major factor in tumour progression in circumstances in which tumour cells are able to outcompete normal cells (Moreno and Basler, 2004; Perez-Garijo et al., 2005; Moreno, 2007).

Acknowledgements

We thank Ainhoa Pérez-Garijo for her contributions during the initial experiments; Ernesto Sánchez-Herrero, Jose Casal and Daniel L. Garaulet for critical comments on the manuscript; Markus Affolter, Konrad Basler, Peter Gallant and Pat Simpson for a key discussion; Tetsuya Tabata and Nicholas Baker for fly stocks; Rick Fehon, Duojia Pan and Herman Steller for antibodies; Angelica Cantarero and Rosa González for general help; and the Servicio de Microscopía Óptica y Confocal of the Centro de Biología Molecular for their help. This work has been supported by grants from the Ministerio de Ciencia e Innovación (Consolider and BFU-02427), from the Comunidad de Madrid and from an institutional grant from the Fundación Ramón Areces. S.C.H. is supported by a JAE-predoc scholarship.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/22/3747/DC1

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Supplementary appendix

To describe the size of the compartments with time in our informatics simulation, we chose the simplest curve commonly used to explain tissue growth: the logistic function, which consists of a symmetrical sigmoid curve with the inflection point in the middle of the size scale. The increment of size in this function is defined as follows (reviewed by Bryant and Simpson, 1984):

$$\frac{dW}{dt} = kW(A - W) \quad [1]$$

where W is the current size (cell number in our case), A the final size and k a constant of growth rate. It should be noted that A is the asymptote of the curve and would only be reached at an infinite point, so a lower value must be used (see below regarding how to estimate the new value of A). But, as long as we are interested not in the net growth of the whole compartment but in that of each individual cell, we can divide this equation between W to describe this:

$$\frac{Growth}{cell} = k(A - W) \quad [2]$$

In this equation, the growth rate of the cell now depends only on how long remains to reach the final size (A-W). In other words, at the beginning of the growing period, when W is irrelevant compared with A, the growth is constant and high. And, as compartment size increases and W gets closer to A, the growth rate of cells decreases. As long as this equation represents the amount of cell cycle progression per time unit, we prefer to use the inverse equation, which represents the cell doubling time (CDT):

$$C.D.T. = \frac{1}{k(A - W)} \quad [3]$$

Fitting the experimentally estimated CDTs to regression lines, the parameters k and A can be calculated for anterior and posterior compartments in both M^+ and M/+ conditions. Thus, the W value is the variable in the equations.

In the simulations in which apoptosis is considered, we made the assumptions that cells are arranged in a honeycomb-like array with six cell contacts per cell (see Gibson et al., 2006) and that clones are isodiametric. This simplification allows us to calculate their perimeter and therefore the number of cells in contact (which potentially could die as a result of cell competition). In this kind of hexagonal array, a new row of cells around a hexagon has a number of cells equal to the number of rows away from the centre multiplied by a factor of 6. We estimate this distance to the centre of a clone with a particular size calculating the clone's radius and adding a factor of 0'5 (because the radius of a circle considers only half of the diameter of the central cell):

$$Cell\ number\ around\ a\ clone = 6 \times (r + 0.5) = 6 \times \left(\sqrt{\frac{A}{\pi}} + 0.5 \right) \quad [4]$$

In which A is the 'area', i.e. the number of cells in the clone in our case.

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